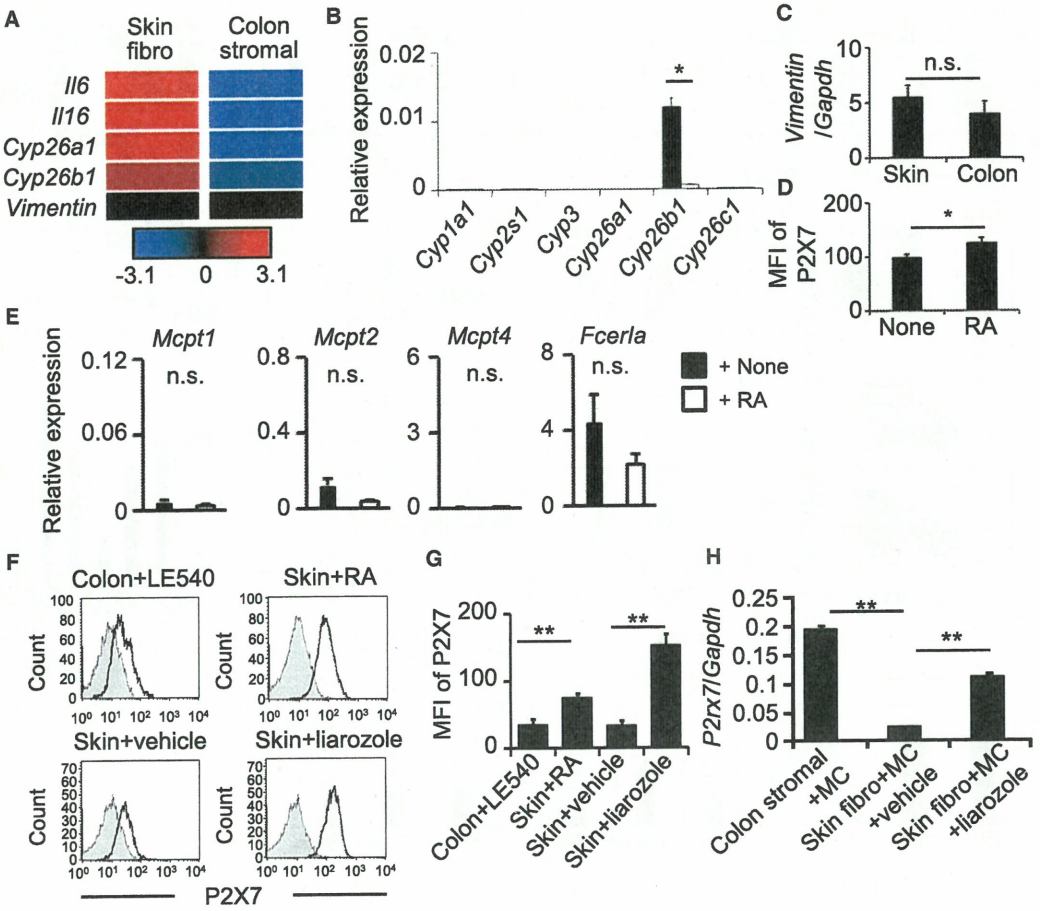

Figure 4. Critical Role of Cyp26b1 in Regulating P2X7 Expression on Mast Cells

(A) Gene microarray analysis was performed to compare the gene expression between skin fibroblasts and colon stromal cells; representative genes are shown.

(B) Gene expression of Cyp26 families was examined by quantitative RT-PCR. Gene expression relative to *Gapdh* is shown. Data are means ± SEM. *$p < 0.05$ ($n = 4$).

(C) Vimentin expression on skin fibroblasts and colon stromal cells was examined by quantitative RT-PCR (qRT-PCR). Relative expressions were normalized against *Gapdh*. Data are means ± SEM ($n = 4$). n.s., not significant.

(D and E) BMMCs were treated with 50 nM of retinoic acid (RA) for 6 days. The expression of P2X7 (D) and *Mcp1*, *Mcp2*, *Mcp4* and *Fcerla* (E) was then examined by flow cytometry and qRT-PCR, respectively. Data are means ± SEM ($n = 4$). *$p < 0.05$.

(F) BMMCs were cocultured with skin fibroblasts or colon stromal cells, with or without RA, LE540, or liarozole and stained for P2X7. Control staining with rat IgG2b is shown as gray. Data are representative of at least three independent experiments.

(G) MFI of P2X7 expression was shown ($n = 3$ to 6). Data are means ± SEM. **$p < 0.01$.

(H) *P2rx7* expression on cocultured BMMCs was examined by qRT-PCR. Data are means ± SEM ($n = 4$). **$p < 0.01$.

P2X7 expression (Heiss et al., 2008). Therefore, it is possible that Cyp26b1-mediated control of RA concentrations by skin fibroblasts regulates P2X7 expression on MCs. To test this hypothesis, we examined the P2X7 expression after adding RA to BMMCs. P2X7 expression increased in the presence of RA without affecting gene expression of *Mcpt1*, *Mcpt2*, and *Mcpt4* (Figures 4D and 4E). Similarly, adding RA to BMMCs in the presence of skin fibroblasts also increased the P2X7 expression (Figures 4F and 4G). Reciprocally, LE540, an inhibitor of retinoid X receptor and retinoid A receptor, suppressed the P2X7 expression on MCs cocultured with colon stromal cells (Figures 4F and 4G). In addition, in vitro treatment of skin fibroblasts with liarozole, a Cyp26b1 inhibitor, augmented P2X7 expression on MCs (Figures 4F–4H). These data demonstrate the critical roles of skin fibroblasts in modulation of P2X7 expression on MCs via the RA metabolic enzyme Cyp26b1.

Aberrant P2X7 Expression on MCs Induces Skin Inflammation, and Its Inhibition Ameliorates Disease Development

Retinoid and its metabolites play important roles in both pro- and anti-inflammatory responses (Fisher and Voorhees, 1996). Retinoids display key physiological roles in maintaining skin homeostasis, and dysregulation of retinoid signaling is found in various skin diseases. Indeed, topical treatment with retinoids causes epidermal hyperplasia and thickening of the differentiated

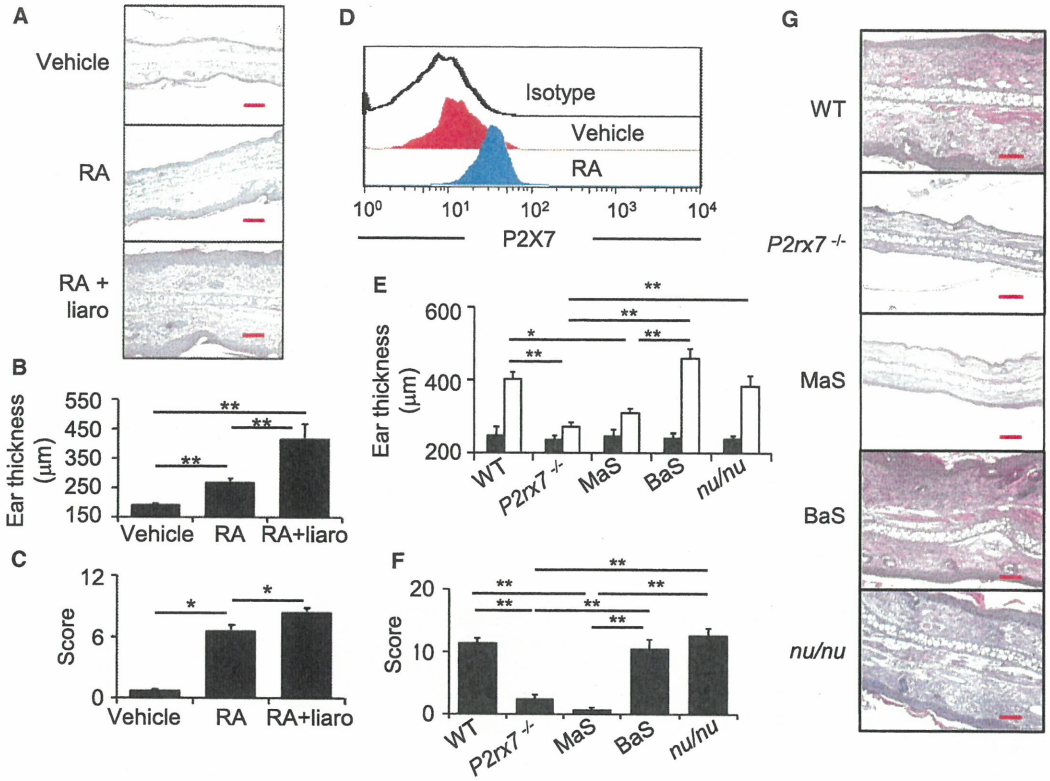

Figure 5. Aberrant P2X7 Expression in Mast Cells Induces Retinoid Dermatitis

(A) Hematoxylin and eosin (H&E) staining of skin from mice treated with vehicle or RA with or without liarozole (liaro) for 2 weeks. Data are representative of at least three independent experiments. Scale bars represent 100 μm.

(B) Thickness of ear was measured. Data are means ± SEM. **$p < 0.01$ ($n = 4$).

(C) Severity of inflammation was scored. Data are means ± SEM. *$p < 0.05$ ($n = 4$).

(D) Expression of P2X7 on skin MCs in RA- or vehicle-treated mice was examined by flow cytometry analysis.

(E) Mice (WT; diphtheria toxin-treated MaS-TRECK, MaS; diphtheria toxin-treated BaS-TECK, BaS; and nude; *nu/nu*) were treated with RA for 8 weeks and ear thickness was measured ($n = 6$ to 24).

(F) Severity of inflammation was scored ($n = 3$ to 4) (means ± SEM. *$p < 0.05$, **$p < 0.01$, one-way ANOVA and Tukey's method).

(G) Representative H&E staining of ear are shown. Scale bars represent 100 μm.

suprabasal layers, eventually leading to retinoid dermatitis (Fisher and Voorhees, 1996; García-Serrano et al., 2011). Additionally, skin irritation occurs with retinoid treatment (Varani et al., 2003). High-dose oral administration of RA to pregnant mice causes skin inflammation in the fetus (Okano et al., 2012). These findings led to a hypothesis that P2X7 expression on skin MCs induced by high concentration of RA might be of relevance to skin irritation or retinoid dermatitis.

To test this hypothesis, mice were orally inoculated with 0.4% RA twice a week for several weeks. These RA-treated mice showed skin irritation and hypertrophy when compared with vehicle-treated mice (Figures 5A–5C; Figure S4A). Histological analysis showed that RA treatment caused dermatitis associated with the accumulation of inflammatory cells and MCs (Figures 5A; Figure S4B–S4E). Importantly, P2X7 expression in skin MCs in RA-treated mice was augmented compared with that in vehicle-treated mice (Figure 5D). Moreover, inhibition of Cyp26b1-dependent RA metabolism by the treatment with liarozole exacerbated the skin inflammation compared with RA treatment alone (Figures 5A–5C; Figure S4B). These results indicated that Cyp26b1 counteract the onset of RA-induced dermatitis. Furthermore, RA-induced dermatitis was ameliorated in *P2rx7*$^{-/-}$- and MC-deficient mice, but not in basophil-deficient mice (Figures 5E–5G).

We confirmed a previous report (Hall et al., 2011) that RA induces the differentiation of both T helper 17 (Th17) and regulatory T cells (Figure S4F). RA treatment also slightly induced P2X7 expression on skin T cells (Figure S4G). Therefore, to evaluate the possible involvement of T cells in the induction of RA-induced dermatitis, we analyzed T cell-deficient nude (*nu/nu*) mice (Figures 5E–5G). We observed ear swelling accompanied by inflammation in *nu/nu* mice (Figures 5E–5G), indicating that, even though RA treatment affected the P2X7 expression in both T cells and MCs, MCs play central roles in the induction of retinoid dermatitis.

To examine the expression of P2X7 ligands in retinoid dermatitis, we next measured the production of extracellular ATP—a major ligand of P2X7—in mice with retinoid dermatitis or mice receiving skin scratch as a control (Kawamura et al., 2012). We detected elevated amounts of ATP in the skin of mice with

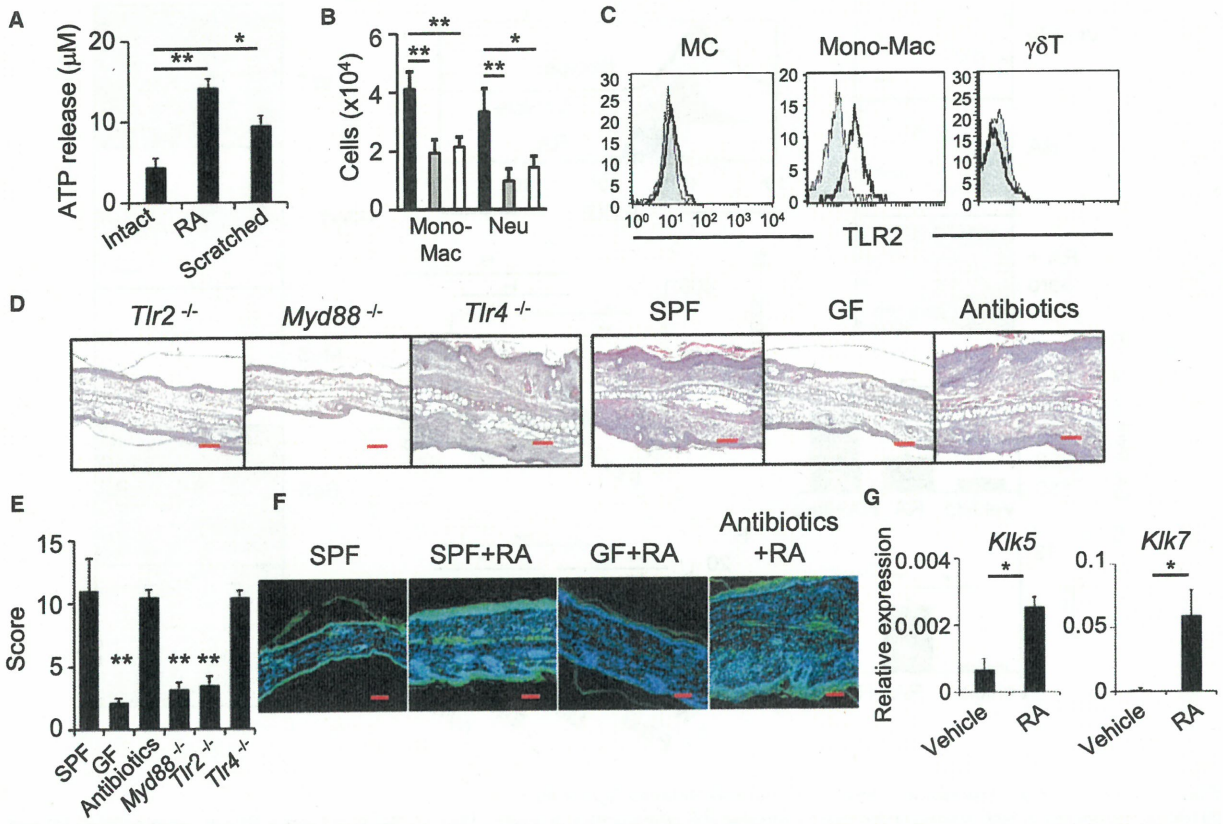

Figure 6. Skin Commensal Bacteria Contribute to P2X7-Medaited Retinoid Dermatitis

(A) ATP concentrations released from skin tissues were measured (means ± SEM. *$p < 0.05$; **$p < 0.01$).

(B) The numbers of monocytes and macrophages (Mono-Mac) and neutrophils (Neu) in the skin after RA-treatment were quantified (black bars, WT; gray bars; *P2x7*$^{-/-}$; white bars, diphtheria toxin-treated MaS-TRECK mice) (means ± SEM. *$p < 0.05$; **$p < 0.01$).

(C) TLR2 expression was examined in MCs, CD11b^{+} Mono-Mac, and γδ T cells. Control staining with isotype control antibody is shown as gray.

(D) *Tlr2*$^{-/-}$, *Myd88*$^{-/-}$, and *Tlr4*$^{-/-}$, specific-pathogen-free (SPF), germ-free (GF), and antibiotic-treated mice were treated with retinoic acid for 8 weeks; representative hematoxylin and eosin staining of ear are shown. Data are representative of at least three independent experiments. Scale bars represent 100 μm.

(E) Severity of inflammation was scored (n = 4 to 6). Data are means ± SEM. **$p < 0.01$; one-way ANOVA and Tukey's method.

(F) Cathelicidin (green) expression was measured by confocal microscopy. Scale bars represent 100 μm.

(G) Kallikrein (KLK) 5 (n = 9) and 7 (n = 4) expression was examined by qRT-PCR. Data are means ± SEM. *$p < 0.05$.

retinoid dermatitis as scratched skin (Figure 6A). In addition, degradation of extracellular ATP by local administration of apyrase suppressed at least partly the severity of retinoid dermatitis (Figures S5A–S5C). The ATP-P2X7 pathway leads to the production of lipid mediators (e.g., leukotriene B4) and chemokines (e.g., MCP1, CCL7, and CXCL2) from MCs to recruit inflammatory cells such as monocytes and neutrophils (Kurashima et al., 2012). We found that CD11b^{+} monocytes and macrophages and Gr-1^{+} neutrophils were increased in the skin of mice treated with RA, but their abundance was lower in RA-treated mice lacking P2X7 or MCs (Figure 6B). These results suggest that increased ATP production in the RA-treated skin caused P2X7- and MC-dependent skin inflammation.

P2X7-Mediated Retinoid Dermatitis Requires Innate Immune Signaling

External stimuli—especially antimicrobial signaling—can provoke or inhibit skin inflammation (Jin et al., 2009). We found that TLR2 was highly expressed by the accumulated cells (e.g., monocytes and macrophages) but relatively low in MCs and γδ T cells (Figure 6C). To determine whether microbial stimulation via TLR2 participated in the induction of retinoid dermatitis, *Tlr2*$^{-/-}$ and *Myd88*$^{-/-}$ mice were inoculated with a high dose of RA. Those mice had only minor incidence of retinoid dermatitis after 8 weeks of RA inoculation, whereas identically treated *Tlr4*$^{-/-}$ mice had typical signs of retinoid dermatitis (Figures 6D and 6E). These results suggest that signaling from TLR2 contributes to the development of retinoid dermatitis. It remains unclear whether TLR2 signaling is mediated by microbial components, because other endogenous ligands, such as hyaluronan fragments, biglycan, and serum amyloid A, also activate TLR2 (Erridge, 2010). Therefore, to determine the requirement for TLR2 ligands derived from skin microbiota, we induced retinoid dermatitis in GF mice. In addition, we used mice receiving antibiotics orally because, unlike GF mice, which were free from both gut and skin microbiota, oral antibiotic administration rarely

influences the skin microbiota but diminishes the gut microbiota (Naik et al., 2012). Whole-mount fluorescent in situ hybridization (FISH) analysis with EUB338 (a probe for most bacterial species) confirmed the presence of skin bacteria in antibiotic-treated mice, but not in GF mice (Figure S5D). When these two groups of mice were subjected to retinoid dermatitis, inflammatory signs were found in the antibiotic-treated mice, but not GF mice (Figures 6D and 6E). These findings suggest that recruitment and activation of $TLR2^+$ monocytes and macrophages by skin microbiota are important for the aggravation of retinoid dermatitis.

Not only ATP but also the cathelicidin-derived peptide LL37 directly activates P2X7 (Elssner et al., 2004). Moreover, bacterial components such as lipoteichoic acid, a ligand of TLR2, induce cathelicidin production (Yamasaki et al., 2007). Indeed, we found that expression of gene encoding *cathelicidin* were low in GF mice subjected to retinoid dermatitis induction, but they were unaltered in antibiotic-treated mice with retinoid dermatitis, compared with SPF mice (Figure 6F). In addition, in vitro analysis revealed that inflammatory monocytes increased the extracellular ATP production and cathelicidin expression via TLR2-dependent manner (Figures S5E and S5F). Kallikrein (KLK) 5 and 7 are required to process cathelicidin to produce LL37 in the skin (Morizane et al., 2010). We found that RA treatment upregulated the expression of both *Klk5* and *Klk7* in the area of retinoid dermatitis (Figure 6G). Furthermore, another in vitro analysis indicated that *Cxcl1* and *Cxcl2* expression was increased in MCs when they were cocultured with monocytes activated via TLR2 (Figure S5G). These observations indicated that cross-communication between skin microbiota- and TLR2-dependent production of P2X7 ligands such as ATP and LL37 from inflammatory monocytes and the activation of MCs via P2X7 is critical for the development of retinoid dermatitis.

DISCUSSION

Skin and mucosa possesses immunologically unique regulatory systems that create balanced homeostatic conditions in the face of the harsh outside environment. It was suggested that tissue environmental factors regulate locally unique MC phenotypes (Xing et al., 2011). As protease expression patterns on MCs (Xing et al., 2011), our previous findings suggested that P2X7 expression on MCs differs between the skin and intestine (Kurashima et al., 2012). Here, we found a function of skin fibroblasts in reducing the P2X7 expression on MCs through the metabolism of RA. This pathway creates unique niches for regulating the homeostatic network of the skin-surface barrier by inhibiting excessive ATP-mediated activation of MCs. We also proposed the possible involvement of LL37 as another ligand of P2X7 in skin inflammation. These observations reveal the unique tissue-dependent immune regulation mediated by structural cells such as stromal cells and fibroblasts to create homeostasis at the skin-surface barrier. Breakdown of this system leads to the development of inflammation such as retinoid dermatitis.

Activation of MCs is tightly regulated by multiple receptors. For instance, elevated expression of IL-33 from epidermal keratinocytes and dermal fibroblasts as a result of sun exposure induces MC activation and hence skin inflammation (Lunderius-Andersson et al., 2012). Simultaneously, various inhibitory receptors, such as leukocyte monoimmunoglobulin-like receptor 3 and paired immunoglobulin-like receptor B, are typically expressed on MCs (Izawa et al., 2012; Masuda et al., 2007). Along with the expression of these inhibitory receptors, regulation of the expression of activation-type receptors such as P2X7 might be important for inhibiting the abnormal activation of MCs under tissue-specific environment, for example at sites where ligands (e.g., ATP and LL37) are constantly or easily produced. Our results showed that TLR2-mediated activation was involved in extracellular ATP release and simultaneous enhancement of LL37 production by monocytes or macrophages. Furthermore, MCs produced ATP via the actions of ATP synthase and adenylate kinase (Kurashima et al., 2012). Thus, extracellular ATP is released from a various sources at millimolar concentration, which are sufficient for P2X7-mediated MC activation at the site of skin inflammation (Takahashi et al., 2013). Therefore, the balanced and optimal expression of activation and inhibitory receptors, as well as ligand production, is required to maintain homeostasis, which was tissue dependent. From this perspective, our findings provide evidence for the presence and mechanism of skin-specific negative regulation of ATP-mediated MC activation via the downregulation of P2X7.

RA has been used clinically to normalize skin homeostasis in patients with acne or psoriasis (Geria and Scheinfeld, 2008). However, topical application of excessive amounts of RA accelerates skin inflammation (Fisher and Voorhees, 1996). Treatment with excessive amounts of 9-*cis* RA causes epidermal proliferation and increased skin thickness in mice (García-Serrano et al., 2011). Furthermore, Cyp26b1 deficiency in keratinocytes and fibroblasts causes skin barrier disruption and inflammation in mice (Okano et al., 2012). Our study indicated the importance of RA-mediated upregulation of P2X7 on MCs in the development of skin inflammation. Liarozole inhibits RA metabolism with targeting not only Cyp26b1 but other P450 enzymes; thus, off-target effects of liarozole could not be negligible (De Coster et al., 1996). Our data indicated the highest expression of Cyp26b1 in skin fibroblasts; these data, plus those from the studies mentioned above, reflect the important aspects and kinetics of the skin fibroblast-Cyp26b1-mediated regulatory and inhibitory system for creating and maintaining a physiologically optimized skin-surface barrier system.

Mcpt expression is reversibly regulated by the tissue environment (Xing et al., 2011). Our data indicate that RA does not regulate Mcpt expression, suggesting that the soluble signals emanating from stromal cells and fibroblasts for MC differentiation and for downregulation of P2X7 expressions differ from each other. Our current findings also revealed that coculture with lung fibroblasts partially reduced P2X7 expression in BMMCs. This effect on P2X7 expression was weaker than that of skin fibroblasts; moreover, P2X7 suppressive capacity was correlated with reduced expression of Cyp26b1 (data not shown). Because concentrations of extracellular ATP were high in respiratory inflammation and lung MCs in vivo expressed high P2X7 (Idzko et al., 2007), reducing P2X7 expression on MCs by enhancing Cyp26b1 expression in fibroblasts in the lung would be a therapeutic strategy for respiratory disorders.

MCs are involved in various skin inflammation, such as psoriasis, atopic dermatitis, and alopecia areata (Cetin et al., 2009; Gilfillan and Beaven, 2011; Otsuka et al., 2011). In this context, high vitamin A concentration in serum is associated with a risk

of atopic dermatitis (Kull et al., 2006). In addition, it was recently reported that dietary vitamin A is involved in aggravating chronic inflammatory status in alopecia both in mice and human (Duncan et al., 2013). In mice with alopecia areata, RA synthesis is increased and simultaneously RA degradation is decreased, thus resulting in excess concentrations of RA at the site of skin inflammation (Duncan et al., 2013). Indeed, P2X7$^+$ MCs are accumulated at the site of alopecia in human (data not shown). These results imply that dysregulation of P2X7 expression on MCs occurs in particular skin inflammation associated with disruption and interruption of RA metabolism. Therefore, it is possible that RA-induced P2X7–MC cascades are also involved in the RA-related skin inflammation.

Skin-resident bacteria play an autonomous role in controlling the local inflammatory milieu by modulating the function of skin-resident T cells (Naik et al., 2012). Functional TLRs are expressed on MCs (Matsushima et al., 2004; Sandig and Bulfone-Paus, 2012). In addition, cathelicidin expression on MCs is induced by the activation of TLR2 by lipoteichoic acid produced by commensal bacteria. Indeed, TLR2 is highly expressed on BMMCs (Wang et al., 2012). Therefore, it is possible that P2X7 and TLR2 dual pathways affect the skin MC-induced production of inflammatory mediators. However, our experiments with three different clones (6C2, mT2.7, and mT2.5) of anti-TLR2 monoclonal antibodies revealed that TLR2 was poorly expressed on the surfaces of skin MCs. Indeed, reconstitution of TLR2-deficient MCs to the MC-deficient mice showed retinoid dermatitis after RA-treatment (data not shown). In contrast, CD11b$^+$ monocytes and macrophages in the skin highly expressed TLR2, indicating that skin-microbiota-mediated signaling stimulates accumulation and activation of inflammatory cells in the skin; this might initiate disruption of skin homeostasis, including the skin fibroblast-Cyp26b1-mediated downregulation system of P2X7-dependent MC activation. In addition, recruitment or activation of the TLR2-expressing inflammatory cells is led by chemokines (e.g., MCP1) or lipid mediators (e.g., leukotrienes) released upon MC activation and the induction of inflammatory responses by ATP release. MCs also directly regulate DC activation in the skin; indeed, maturation and migration of DCs are regulated by TNF-α and ICAM1 on MCs; thus, MC-DC direct interaction could also affect the pathogenesis of skin inflammation (Otsuka et al., 2011). Our in vitro observation revealed that TLR2$^+$ inflammatory cells directly enhanced CXCL1 and CXCL2 expression in MCs, which potentially led to the neutrophil recruitment into the inflammatory site. These results implied the existence of the inflammatory loop among various immune cells including MCs.

These findings collectively reveal that MCs in various tissues help to create tissue-specific local homeostasis operated by individual structural cells (e.g., fibroblasts). Cyp26b1-expressing skin fibroblasts thus play a central role in homeostatic P2X7 downregulation on skin MCs, leading to the suppression of ATP-induced abnormal activation. When the skin homeostatic pathway is sporadically interrupted, such as in hypervitaminosis A, skin bacterial stimulation triggers inflammation via the TLR2-mediated pathway. Our additional understanding of the molecular and cellular bases of the functions and phenotypes of localized MCs and their surrounding structural cells (e.g., fibroblasts and stromal cells) will facilitate the discovery of innovative and beneficial targets for the control of tissue-specific inflammation.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, Balb/c, GF, and *nu/nu*$^{-/-}$ mice were purchased from Japan CLEA. *Rag1*$^{-/-}$, *Tcrb*$^{-/-}$, *Tcrd*$^{-/-}$, *Ighm*$^{-/-}$, *P2rx7*$^{-/-}$, and *Itgax*-DTR mice were obtained from Jackson Laboratory. *Tlr2*$^{-/-}$, *Tlr4*$^{-/-}$, and *Myd88*$^{-/-}$ mice were obtained from Dr. S. Akira (Osaka University). *Id2*$^{-/-}$ mice were generated as previously described (Yokota et al., 1999). *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice were obtained from Dr. H. Suto (Juntendo University). MaS-TRECK and BaS-TRECK mice were established as previously reported (Sawaguchi et al., 2012). All mice were maintained under SPF or GF conditions at the Experimental Animal Facility of the Institute of Medical Science, the University of Tokyo. All experiments were approved by the Animal Care and Use Committee of the University of Tokyo.

In Vivo Treatment

To remove DCs, we intraperitoneally treated *Itgax*-DTR mice with DT (500 ng, Sigma-Aldrich) (Jung et al., 2002). To deplete MCs and basophils, we injected MaS-TRECK and BaS-TRECK mice intraperitoneally with 250 ng DT for 5 consecutive days and then with 150 ng DT every other day (Sawaguchi et al., 2012). Mice were orally inoculated with 0.4% RA (Sigma-Aldrich) in corn oil (Wako) twice a week. Liarozole (1 mg, intraperitoneally) or apyrase (1 U/ear, grade VII, Sigma-Aldrich, intradermally) was injected before RA treatment. Mice received a mixture of ampicillin (1 g/L; Sigma-Aldrich), vancomycin (500 mg/L; Shionogi), neomycin (1 g/L; Sigma-Aldrich), and metronidazole (1 g/ L; Sigma-Aldrich) in drinking water since 2 weeks before RA treatment (Obata et al., 2010). MCs were reconstituted in *Kit*$^{W\text{-}sh/W\text{-}sh}$ mice by intraperitoneal and intravenous (5 × 10^6 cells each) or intradermal (1 × 10^6 cells) injection of BMMCs.

Cell Preparations

Mononuclear cells from the small intestine, colon, PEC, lung, and skin were isolated, and BMMCs were obtained as previously described (Kurashima et al., 2012; Kurashima et al., 2007). To prepare stromal cells and fibroblasts, tissues were digested with EDTA and collagenase (Sigma-Aldrich) and adherent cells were passaged every 4 days (process repeated once). Before coculture with BMMCs, cells were treated with 10 ng/ml mitomycin C (Sigma-Aldrich) for 3 hr and washed twice with PBS. In some experiments, BMMCs were cytospinned (600 rpm, 4 min) and stained for 1 hr with alcian blue (Muto Pure Chemicals, Tokyo, Japan) and for 20 min with safranin (Muto Pure Chemicals). Inflammatory monocytes or macrophages were collected from PEC 3 days after intraperitoneal injection of 2 ml of 4% thioglycolate.

In Vitro Treatment

For MCP1 and TNF-α measurement, 2 × 10^5 cells were stimulated with 0.5 mM of ATP for 2 days. For IL-1β measurement, 2 × 10^5 cells were stimulated with 0.1 μg/mL LPS for 8 hr, followed by 0, 0.5, or 5 mM of ATP for 1 hr. Chemokine and cytokine production in the culture supernatant was measured with CBA inflammatory cytokine kit (BD Biosciences) and IL-1β ELISA (R&D Systems). To stimulate TLR2-mediated pathway, we cultured thioglycolate-induced monocytes and macrophages with Pam2CSK4 (0.2 μg/mL, InvivoGen). To measure ATP secretion from skin organ, we floated skin (8.0 mm square) with the epidermis side upward on 12-well plates containing 1 ml PBS and incubated on ice for 10 min. ATP concentration in the culture was measured by ATP luciferase assay (PerkinElmer) (Kawamura et al., 2012; Kurashima et al., 2012).

Flow Cytometry

Cells were incubated with 5 μg/ml of anti-CD16/32 antibody (Biolegend) for 5 min and then stained for 30 min at 4°C with fluorescence-labeled antibodies; c-kit (0.2 μg/mL), Gr-1 (0.4 μg/mL), CD4 (1 μg/mL), CD11b (0.2 μg/mL), CD11c (0.4 μg/mL), and B220 (0.4 μg/mL) (BD Biosciences); FcεRIα (0.4 μg/mL) and CD207 (0.4 μg/mL) (eBioscience); and F4/80 (20 μg/mL) and ER-TR7 (5 μg/mL) (Abcam), and P2X7 (2 μg/mL, clone 1F11) (Kurashima et al., 2012). Flow cytometric analysis and cell sorting were performed with FACSCalibur and FACSAria (BD Biosciences), respectively.

Histology and Scoring

Skin samples were fixed in 4% paraformaldehyde and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin (Wako). To